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Pulse Radiolysis Studies on the Reaction of the Reduced Vitamin B₁₂ Complex Cob(II)alamin with Superoxide

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Reactive oxygen and nitrogen species (ROS/RNS) including hydroxyl radicals, nitric oxide, hydrogen peroxide, peroxynitrite and superoxide, are important signaling molecules and are also generated at high concentrations as part of the innate immune response to pathogens.^[1] However, elevated levels of reactive species can lead to oxidative stress, resulting in irreversible damage to biomolecules.^[1] Oxidative stress has been implicated in numerous diseases including cardiovascular disease, cancer, neurological diseases and aging in general.^[1-2] This has led to considerable interest in the development of therapeutics that scavenge ROS/RNS, including porphyrin-based complexes.^[3]

The structurally related vitamin B₁₂ derivatives cobalamins (Cbls; X = Me or Ado, Scheme 1), are cofactors for two enzyme reactions in humans – cytosolic methionine synthase (MS), and mitochondrial L-methylmalonyl-CoA mutase (MM-CoA mutase).^[4] Cbl supplementation has been shown to be beneficial for treating chronic inflammatory diseases including chronic fatigue syndrome, sepsis, asthma, rheumatoid arthritis, neurological diseases including Alzheimer's disease, and viral based diseases including AIDS.^[5] Recently Scalabrino et al demonstrated that Cbl attenuates the pro-inflammatory immune response in a rat model for chronic Cbl deficiency by modulating levels of cytokines and growth factors including TNF- α , nerve growth factor, epidermal growth factor and IL-6.^[6]

The important ROS superoxide, O₂^{•−}, is produced as a byproduct of cellular metabolism by mitochondrial and reticular membrane electron transport systems and by enzymes such as NADPH oxidase and xanthine oxidase.^[7] O₂^{•−} is a precursor of two of the most potent ROS/RNS, peroxynitrite and the hydroxyl radical.^[8] Superoxide dismutases, enzymes that catalyze the destruction of O₂^{•−} by converting it to O₂ and H₂O₂, are upregulated in oxidative stress - associated diseases.^[1] *In vitro* cell studies using human aortic endothelial cells show that vitamin B₁₂ prevents O₂^{•−} - induced oxidative stress at physiologically relevant (~10^{−9}M) concentrations.^[9] With supplementation intracellular Cbl levels can reach 0.7 μ M.^[10] Upon entering cells vitamin B₁₂ derivatives (cob(III)alamins) are reduced by the cblC protein to cob(II)alamin, Cbl(II).^[11] We have previously shown that O₂^{•−} generated by the xanthine oxidase/acetaldehyde system reacts rapidly with the radical complex Cbl(II) by an indirect competition method.^[9b] Given the importance of this

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reaction in human health, we now report the direct determination of rate constants for the reaction between Cbl(II) and $O_2^{\cdot-}$ using pulse radiolysis to generate $O_2^{\cdot-}$.

Figure 1 gives a plot of absorbance at 530 nm versus time for the reaction between Cbl(II) ($7.3 \times 10^{-5}M$) and $O_2^{\cdot-}$ ($9.0 \times 10^{-6}M$) at pH 7.42. Irradiating aqueous solutions generates $\cdot OH$, e_{aq}^- and $\cdot H$,^[12] and in O_2 -saturated aqueous solutions containing ethanol or formate, these primary radicals are rapidly converted to $O_2^{\cdot-}$.^[12] An anaerobic Cbl(II) solution was rapidly mixed with an O_2 -saturated aqueous ethanol solution using a stopped-flow mixing device. The resulting mixture was subsequently exposed to an intense ionizing pulse (30 – 300 ns) and data collected. The time between mixing and pulsing was typically <2 s and the Cbl(II) concentration was kept at least 5 times higher than the $O_2^{\cdot-}$ concentration, to achieve essentially pseudo-first order conditions. Our earlier stoichiometry experiments showed that the reaction between Cbl(II) and $O_2^{\cdot-}$ proceeds according to eq (1), with clean oxidation of the metal center of Cbl(II) to give aquacobalamin/hydroxycobalamin, $H_2OCbl^+/HOCbl$ ($pK_a(H_2OCbl^+) = 7.8$)^[13].^[9b]

The data in Figure 1 fit well to a first-order rate equation, giving an observed rate constant, $k_{obs} = (2.70 \pm 0.02) \times 10^4 s^{-1}$. The rate constant is unchanged when data are collected at 435 nm ($k_{obs} = (2.69 \pm 0.05) \times 10^4 s^{-1}$; Figure S1, Supporting Information).

Rate constants for the reaction between Cbl(II) and $O_2^{\cdot-}$ were also determined at other Cbl(II) concentrations at pH 7.42 and the data are summarized in Figure 2. The data fit well to a straight line passing through the origin, consistent with a single irreversible reaction. The linear relationship suggests that the reaction is first-order with respect to Cbl(II) and $O_2^{\cdot-}$. From the slope, the second-order rate constant (k_{app}) of the reaction was $(3.78 \pm 0.07) \times 10^8 M^{-1} s^{-1}$. Separate pulse radiolysis experiments showed that $H_2OCbl^+/HOCbl$ does not react with $O_2^{\cdot-}$ (pH 7.42, 0.500 M EtOH, $I = 0.01$ M (phosphate buffer)). Analyzing this latter data at 260 nm gave a rate constant for $O_2^{\cdot-}$ decomposition similar to that reported in the literature at this pH value (decomposition of superoxide is pH dependent).^[12] Values of k_{app} were also determined at pH 5.50 and 8.72 at a single Cbl(II) concentration, giving $k_{app} = (4.23 \pm 0.18) \times 10^8 M^{-1} s^{-1}$ and $(4.40 \pm 0.22) \times 10^8 M^{-1} s^{-1}$, respectively (Figures S2 and S3, Supporting Information). Given the similarity in these rate constants to the value obtained at pH 7.42 ($3.78 \pm 0.07) \times 10^8 M^{-1} s^{-1}$), k_{app} is essentially independent of pH in this pH range.

It is well established that Cbl(II) is oxidized to $H_2OCbl^+/HOCbl$ by air.^[9b] In order to confirm that Cbl(II) is also oxidized to $H_2OCbl^+/HOCbl$ by $O_2^{\cdot-}$ in our pulse radiolysis experiments, a plot of change in extinction coefficient versus wavelength for the reaction of Cbl(II) with $O_2^{\cdot-}$ was generated and compared with the change in extinction coefficient for the Cbl(II)/($H_2OCbl^+/HOCbl$) conversion, Figure 3. (Note that it was not possible to obtain full spectra as a function of time from our experimental setup for the pulse radiolysis experiments.) There is excellent agreement between the two sets of data and the isosbestic points observed at 375, 490 and 578 nm correspond to the expected values reported for the Cbl(II)/($H_2OCbl^+/HOCbl$) conversion.^[9b] The corresponding plots of absorbance of the reaction product solution versus wavelength for both sets of data are also in excellent agreement with each other (Figure S4, Supporting Information). The formation of $H_2OCbl^+/HOCbl$ from the reaction between Cbl(II) and $O_2^{\cdot-}$ was additionally confirmed by HPLC analysis of the product solution from the pulse radiolysis experiments and by using the xanthine oxidase/acetaldehyde system to generate $O_2^{\cdot-}$ (details given in the Supporting Information).

The amount of H₂O₂ produced in the reaction between Cbl(II) and O₂^{•-}, eq (1), was found to be ~0.96 mol equiv. using the H₂O₂-specific Amplex Red assay from a calibration plot of fluorescence intensity of oxidized Amplex Red versus H₂O₂ concentration.^[14] O₂^{•-} was generated using the xanthine oxidase/acetaldehyde system for these experiments. Details are given in the Supporting Information.

To summarize, the rate constant of the reaction between Cbl(II) by O₂^{•-} has been directly determined using pulse radiolysis and was found to be $3.8 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ (pH 7.42, RT). This value is in reasonable agreement with our earlier rate constant value ($7.0 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$, 0.010 M phosphate buffer, pH 7.40, 25 °C),^[9a] which was determined indirectly using a competitor and generating O₂^{•-} using the xanthine oxidase/acetaldehyde system. Cbl(II) is cleanly oxidized to H₂OCbl⁺/HOCbl and the rate of the reaction is independent of pH in the pH 5.52-8.72 region, as expected, since neither Cbl(II) nor O₂^{•-} ionize in this region. The rate constant for the reaction between Cbl(II) and O₂^{•-} is within an order of magnitude as that reported for cytosolic Cu/Zn superoxide dismutase (Cu/Zn SOD; $2.0 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, pH 5.0-9.5, 25.0 °C^[15]) and mitochondrial manganese superoxide dismutase (MnSOD; $1.1 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ pH 8.2, RT^[16]). Cob(II)alamin is a major intracellular cobalamin form and both intracellular cobalamin and SOD concentrations can reach micromolar levels.^[11b, 17] Intracellular reductases subsequently reduce H₂OCbl⁺/HOCbl to Cbl(II).^[11] Cell studies also show that Cbl prevents O₂^{•-} - induced oxidative stress.^[9] Given these latter results combined with the similarity of intracellular concentrations of Cbl and SOD and the rate constants for the reactions of SOD or Cbl(II) with O₂^{•-}, this supports a role for Cbl as a O₂^{•-} scavenger in addition to acting as a cofactor for the two mammalian B₁₂-dependent enzyme reactions. Since a significant proportion (up to 30-40%) of the elderly population are vitamin B₁₂ deficient^[18] and vitamin B₁₂ is non-toxic even at high doses,^[18] B₁₂ supplementation may be especially beneficial for treating chronic inflammatory diseases associated with aging. Enzyme-bound Cbl(II) is also likely to be rapidly oxidized to its inactive H₂OCbl⁺/HOCbl form by O₂^{•-}, consistent with inactivation of the B₁₂enzymes under oxidative stress conditions.^[19]

Experimental Section

Experimental details are included in the Supporting information section.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Plot of change in absorbance at 530 nm versus time for the reaction of $O_2^{\cdot-}$ ($9.0 \times 10^{-6} M$) with excess Cbl(II) ($7.3 \times 10^{-5} M$) at pH 7.42 (0.500 M EtOH, $5.00 \times 10^{-3} M$ phosphate buffer, $I = 0.010 M$, RT). The best fit of the data to a first-order rate equation is superimposed on the data, giving an observed rate constant, $k_{obs} = (2.70 \pm 0.02) \times 10^4 s^{-1}$.

Figure 2.

Plot of k_{obs} versus Cbl(II) concentration for the reaction between excess Cbl(II) ($(1.6 - 7.3) \times 10^{-5} M$) and $O_2^{\cdot -}$ ($(1.0 - 9.0) \times 10^{-6} M$) at pH 7.42 (0.500 M EtOH, $5.00 \times 10^{-3} M$ phosphate buffer, $I = 0.01 M$, RT). Points represent the mean value of at least three independent measurements at three different wavelengths. Data have been fitted to a line passing through the origin, giving $k_{app} = (3.78 \pm 0.07) \times 10^8 M^{-1} s^{-1}$.

Figure 3. Change in the molar absorption coefficient versus wavelength for the reaction of (■) excess Cbl(II) ($(1.6 - 7.3) \times 10^{-5} \text{M}$) with $\text{O}_2^{\cdot-}$ ($(1.0 - 9.0) \times 10^{-6} \text{M}$) at pH 7.42 (0.500 M EtOH, $5.00 \times 10^{-3} \text{M}$ phosphate buffer, $I = 0.01 \text{ M}$, RT) and (●) Cbl(II) ($5.00 \times 10^{-5} \text{M}$) with O_2 (from air), to form $\text{H}_2\text{OCbl}^+/\text{HOCbl}$ ($5.00 \times 10^{-3} \text{M}$ phosphate buffer, 0.500 M EtOH, $I = 0.01 \text{ M}$, RT). Inset: UV-vis spectra for the oxidation of Cbl(II) ($3.00 \times 10^{-5} \text{M}$) by O_2 , to form $\text{H}_2\text{OCbl}^+/\text{HOCbl}$ ($5.00 \times 10^{-3} \text{M}$ phosphate buffer, 0.500 M EtOH, $I = 0.01 \text{ M}$, RT).

Scheme 1.

Structure of vitamin B₁₂ derivatives (cobalamins). X = CH₃, Ado, H₂O/OH, NO, etc.